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Toxicology Letters 160 (2006) 151–157
Toxicology Letters
www.elsevier.com/locate/toxlet

Androgen receptor activities of *p*,*p*′-DDE, fenvalerate and phoxim detected by androgen receptor reporter gene assay

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Received 23 May 2005; accepted 27 June 2005
Available online 25 August 2005

Abstract

In this study, we have developed a transient human androgen receptor (hAR) reporter gene assay using African monkey kidney cell line CV-1. The assay displayed appropriate response to the known androgen receptor (AR) agonist 5α-dihydrotestosterone (DHT) and AR antagonist nilutamide. DHT induced AR-mediated transcriptional activity in a concentration-dependent manner with median effective concentration (EC_{50}) value of 3.90×10^{-10} M. Nilutamide exhibited potent antiandrogenic activity with median inhibitory concentration (IC_{50}) value of 8.90×10^{-7} M. The transcription could not be activated by glucocorticoid receptor (GR) agonist dexamethasone, which suggested that the assay system be highly specific to androgenic compounds without cross-talk to GR agonist. The assay shows acceptable repeatability to DHT with intra coefficient of variability (CV) of 9.3% and inter CV of 10.9%. We applied this assay to evaluate the androgenic and antiandrogenic activities of some pesticides including organochlorine *p*,*p*′-dichlorodiphenyldichloroethylene (DDE), pyrethroid fenvalerate and organophosphorus phoxim. The results showed that *p*,*p*′-DDE exhibited potent antiandrogenic activity. Fenvalerate acted as weak AR antagonist and phoxim did not show AR antagonistic activity. We failed to find AR agonistic effects of the three pesticides. The present study provided a promising tool to screen for AR agonists and antagonists.

Keywords: Reporter gene assay; Human androgen receptor; African monkey kidney cell; Antiandrogenic activity; Pesticide

1. Introduction

Concerns have been raised about endocrine disrupting chemicals (EDCs) that provide a potential threat to human and wildlife. It has been confirmed that EDCs can disturb sexual characteristics in wildlife populations such as fish (Gimeno et al., 1996) and amphibians (Hayes et al., 2002). The exposure to EDCs might contribute to the reported decline in sperm counts (Swan et al., 2000), increase in hypospadias, cryptorchidism and testicular cancer in males (Paulozzi, 1999) as well as increase in spontaneous abortion and endometriosis in females (Koninckx, 1999). A major mechanism of endocrine disrupting is mimicking or inhibiting the steroid hormone by directly binding to the hormone receptors either as agonists or antagonists (Waters et al., 2001). Environmental estrogens have been the focus of the majority of researches in the last 20 years (Miller et al., 2001; Takeyoshi et al., 2002). Recently, several chemicals have been demonstrated antiandrogenic activity by interfering with androgen receptor (AR)

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doi:10.1016/j.toxlet.2005.06.016

(Kelce et al., 1994, 1995; Lambright et al., 2000; Sohoni et al., 2001; Paris et al., 2002; Vinggaard et al., 2002).

Pesticides are commonly used for the control of agricultural and indoor pests. The organochlorine pesticides such as chlorodiphenyltrichloroethane (DDT) were wide used from 1960s to 1980s. Although the use of DDT has been banned in the United States since 1972 and in China since 1983, it continues to be a potential health hazard for the general population because of its worldwide use in the past years, its accumulation in the food chain, and its long half-life in human issues and sediments due to its lipophilic nature (Turusov et al., 2002). The primary metabolite of DDT, *p*,*p*′-dichlorodiphenyldichloroethylene (DDE) has been detected in soils, plant tissues, benthic species and human tissues (Waliszewski et al., 2004; Wolff et al., 2005). Since organochlorine insecticides restricted, the organophosphate and pyrethroid pesticides have become the most popular pesticides used in the world.

Some pesticides are the most likely suspects as EDCs known to induce estrogenic effects by binding to ER (Andersen et al., 2002; Chen et al., 2002). In contrast, we know comparatively little about pesticides exerting antiandrogenic activities through interaction with androgen receptor (AR). To date, vinclozolin, *p*,*p*′-DDE, linuron and fenitrothion have been reported as antiandrogenic pesticides (Kelce et al., 1994, 1995; Lambright et al., 2000; Sohoni et al., 2001). The publications of pesticides with antiandrogenic activities expand the research effort to screen for pesticides including organochlorine, organophosphate and pyrethroid capable of interfering with AR.

Our laboratory has focused on reproductive effects of some pesticides including the pyrethroid pesticide fenvalerate and the organophosphate pesticide phoxim (Xu et al., 2004; Xia et al., 2004, 2005; Bian et al., 2004). It was shown that fenvalerate and phoxim induced testicular lesions and sperm motility changes in the rats. Fenvalerate exposure was associated with sperm morphologic abnormality and genotoxic effects in human. Because AR is an important factor in male reproduction, molecular mechanism responsible for these adverse effects may be consistent with the inhibition of AR binding and subsequent AR-dependent transcription.

The United States Environmental Protection Agency (US EPA) proposed the receptor reporter gene assays for inclusion in a Tier 1 screening battery (T1S) to detect EDCs acting as receptor agonists and antagonists (EDSTAC, 1998). Some investigators have developed AR reporter gene assays (Gaido et al., 1997; Vinggaard et al., 1999; Terouanne et al., 2000; Euling et al., 2002; Wilson et al., 2002). The assays are designed to identify substances that might interfere with normal androgen activity through ligand–AR interaction. The assays provide a relatively simple way to indirectly reveal whether a substance can activate or inhibit the transcriptional activation of AR-regulated genes by the measurement of the reporter gene product, typically an enzyme or a protein.

The AR reporter gene assays are based on the same general principles, but often use different cell lines including yeast cells (Gaido et al., 1997; Sohoni and Sumpter, 1998) and mammalian cells. During the last few years, mammalian cell lines have been increasingly used to measure AR induced transcriptional activation. The MDA-MB-453 cell is chosen in the AR reporter gene assays because the cell has been shown to constitutively express AR (Wilson et al., 2002). However, the cell also contains glucocorticoid receptor (GR) which shares common hormone responsive element (HRE) with AR. So, though the AR reporter gene assay using MDA-MB-453 cell is useful in screening for both AR and GR agonists, the specificity of the assay is low. The assays employing the cells that do not express steroid receptors provide greater specificity in detecting AR-mediated responses (Hartig et al., 2002). The African monkey kidney cell line CV-1 constitutes an excellent tool in the AR reporter gene assays which does not express endogenous steroid receptors, and thus has a very low background in these assays.

In this study, we have developed a transient reporter gene assay for human androgen receptor (hAR) based on CV-1 cell. The assay was applied to evaluate the antiandrogen activities of some pesticides including *p*,*p*′-DDE, fenvalerate and phoxim.

2. Materials and methods

2.1. Chemicals

Nilutamide (purity >99%), 5α-dihydrotestosterone (DHT, purity >99%) and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The pesticides *p*,*p*′-DDE, fenvalerate and phoxim (purity >99%) were purchased from Fluka (Fluka Chemie GmbH, CH-9471 Buchs, Switzerland). Chemical structures of the three pesticides were shown in Fig. 1. Stock solutions of the chemicals were prepared in absolute ethanol at a concentration of 10^{-2} M, stored at −20 °C, and diluted to desired concentrations in phenol red-free RPMI1640 medium (Sigma Chemical Co.) immediately before use. The final ethanol concentrations in the

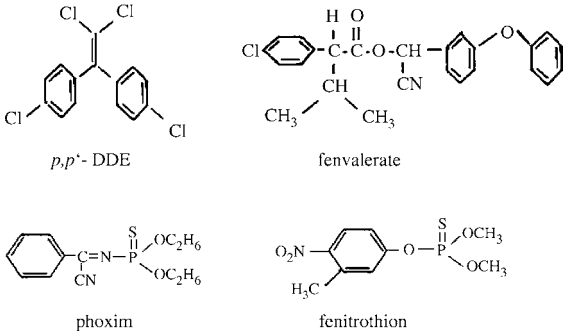

Fig. 1. Chemical structures of some pesticides. The three pesticides *p,p′*-DDE, fenvalerate and phoxim were used in this study.

culture medium did not exceed 0.1% (v/v) that did not affect cell yields.

2.2. Plasmids

We constructed the reporter plasmid mouse mammary tumor virus (pMMTV)-CAT based on the pCAT3-Basic vector (Promega, Madison, WI, USA). The oligonucleotides of MMTV sequences containing four ARE sequences and a TATA promoter were synthesized and inserted into the *Kpn* I and *Bgl* II sites of pCAT3-basic to construct the plasmid pMMTV-CAT. The plasmid pSV-β-galactosidase (pSV-β-Gal, used as internal control for transfection efficiency and cytotoxicity of the test substances) was purchased from Promega (Madison, WI, USA). The hAR expression plasmid AR/pcDNA3.1 containing the full open reading frame of hAR cDNA was the kind gift from Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The plasmid was constructed as described (Takeyoshi et al., 2003).

2.3. Cell culture and tranfection

The CV-1 cell line was obtained from Institute of Biochemistry and Cell Biology in Shanghai, Chinese Academy of Science. The cells were maintained in phenol red-free RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2/air. The host cells were plated in 6-well microtiter plates (Nunc, Denmark) at a density of 1.0×10^5cells per well in the RPMI1640 medium containing 10% charcoal-Dextran-stripped FBS (CDS-FBS). After 24 h, each well was tranfected with 4.0 μg pMMTV-CAT, 0.4 μg AR/pcDNA 3.1 and 1.0 μg pSV-β-Gal using the ESCORT V Transfection Reagent (Sigma Chemical CO.). After an incubation period of 24 h, the transfection medium was removed and various concentrations of DHT and test chemicals dissolved in medium were added for measurement of agonistic activity. In order to measure the AR antagonistic activity, 0.5 nM DHT was added along with the test chemicals. Vehicle control wells contained medium with 0.1% ethanol.

2.4. Reporter gene assays

The cells were harvested 24 h after dosing. After rinsed three times with phosphate-buffered saline (PBS, pH 7.4), the cells were lysed with 1× lysis buffer (Promega, Madison, WI, USA, 400 μl/well). After centrifuged at 12 000 × *g* for 5 min to remove debris, the cell lysates either analyzed immediately or quick-frozen at −70 °C. CAT was measured with the commercial CAT-ELISA (enzyme-linked immunosorbent assay) kit (Roche Molecular Bioch, Mannheim, Germany) following the manufacturer's instructions. Additional aliquots of cell lysates were assayed to determine β-Gal activity using the β-Gal Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI, USA). The amount of CAT for each lysate was normalized to the β-Gal activity. The relative transcriptional activity was converted to fold induction above the vehicle control value (*n*-fold).

2.5. Statistical analysis

The values shown were mean ± S.D. from three independent experiments with tripplicate wells for each dose. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons test when appropriate. The level of significance was set at $p < 0.05$. For agonists, treatments were compared to the vehicles control group; while for androgen antagonists, treatments were compared to the DHT positive control groups.

3. Result

3.1. Response to a known AR agonist DHT

DHT, known as AR agonist, was assayed over concentrations ranging from 10^{-12} to 10^{-6} M. The chemical induced CAT activity in a concentration-dependent manner (Fig. 2). The CAT amount significantly increased at 10^{-10} M. The maximal induction of 79.30-fold of vehicle control achieved at 10^{-8} M and remained at the plateau level thereafter. From the dose–response curve, the median effective concentration (EC_{50}) value of DHT was 3.9×10^{-10} M. When the CV-1 cells were not

transfected with AR expression plasmid AR/pcDNA3.1, the CAT amount was 0.20 ± 0.021 normalized CAT unit. Following DHT administrated, the CAT amount was not significantly changed with the value of 0.20 ± 0.026 normalized CAT unit. It is demonstrated that DHT was not able to induce a transactivation response in the absence of AR. DHT was suggested to induced CAT activity in an AR-depended manner. Analysis of responses of six replicates shows that 10^{-8} M DHT induced CAT activity with an intra-assay (within plate) coefficient of variability (CV) of 9.3% and the inter-assay (across all six plates) CV of 10.9%.

3.2. Response to a known GR agonist dexamethasone

Since AR and GR shared common HRE in the MMTV promoter, dexamethasone was tested to access assay specificity. The CAT activity could not be induced by dexamethasone at the tested dosages from 10^{-10} to 10^{-5} M (Fig. 2). It was suggested that the assay system was highly specific without cross-talk to GR agonist.

3.3. Response to a known AR antagonist nilutamide

To characterize the response of the assay system to antiandrogen, a known AR antagonist nilutamide was coadministered with 0.5 nM DHT in the medium. The dose of DHT was chosen because it was within the linear response range of the dose–response curve of DHT. As expected, nilutamide was a potent antiandrogen that significantly inhibited the CAT activity induced by 0.5 nM DHT at concentration of 10^{-7} M and greater (Fig. 3). The median inhibitory concentration (IC_{50}) value was 8.90×10^{-7} M.

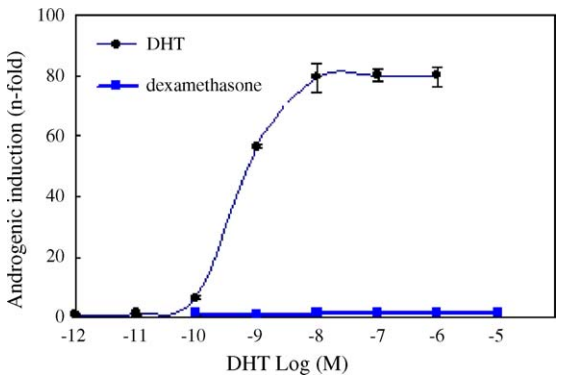

Fig. 2. Dose–response curves of DHT and dexamethasone in the AR reporter gene assay. After cotransfected, the CV-1 cells were treated with increasing concentrations of the chemicals. Values are mean ± S.D.

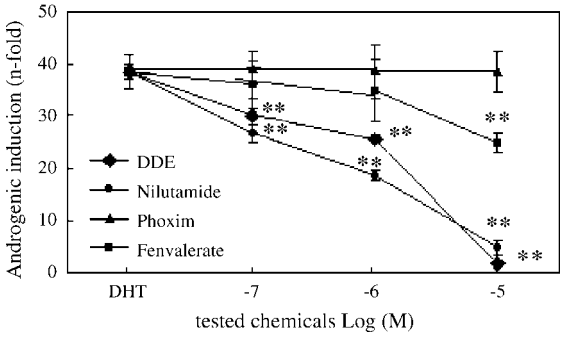

Fig. 3. AR antagonistic activity of nilutamide, *p,p′*-DDE, fenvalerate and phoxim in the AR reporter gene assay. After cotranfected, the CV-1 cells were incubated in the presence of 0.5 nM DHT with increasing concentrations of the compounds. Values are mean ± S.D. $^{**}P < 0.01$ compared with DHT positive control values.

3.4. Androgenic and antandrogenic activities of the pesticides

To avoid cytotoxicity caused by the pesticides, the concentrations of the pesticides were performed at $\leq 10^{-5}$ M. No cytotoxicity was detected in the assay. None of the three pesticides exhibited transcriptional activities at the tested concentrations from 10^{-7} to 10^{-5} M (data not shown).

We tested the pesticides for their inhibitory effect on the CAT activity induced by 0.5 nM DHT. When *p,p′*-DDE was coadministered with DHT, the antagonistic activity was apparent. At concentrations of 10^{-7} M and greater, *p,p′*-DDE significantly inhibited DHT induced CAT activity with IC_{50} of 6.13×10^{-7} M. Fenvalerate did not reduce DHT induced CAT activity at 10^{-7} M, while significant decreases of CAT activity were detected at the higher concentrations between 10^{-6} and 10^{-5} M. The IC_{50} value of fenvalerate was 7.10×10^{-5} M. No significant antagonistic activity was detectable at the tested dosages for phoxim (Fig. 3).

4. Discussion

To cope with the growing numbers of EDCs with antiandrogenic properties, the AR reporter gene assays were developed in many laboratories (Gaido et al., 1997; Vinggaard et al., 1999; Terouanne et al., 2000; Euling et al., 2002; Wilson et al., 2002). However, relative to ER reporter gene assays, test systems measuring AR agonistic and antagonistic effects in vitro are still few. In addition, currently, there are no generally accepted standardized methods for these assays. In order to test the accuracy and comparability of existing assays, Korner et al. (2004) evaluated and compared four in vitro assays

for the detection of AR-mediated agonists and antagonists in an international interlaboratory study including Germany, Denmark, France and USA. Three of four laboratories worked with AR reporter gene assays that have been described previously (Terouanne et al., 2000; Vinggaard et al., 1999; Wilson et al., 2002). This study was not performed to test which assay was best for detecting androgen agonists and antagonists, because in choosing an assay system to use, many factors including the equipment of laboratory and cost-effectiveness must be taken into account. So, if proved to be sensitive to detect known AR agonists and antagonists with reasonable accuracy, the assays can be adopted in the screening process for the androgens and antiandrogens.

In this study, we developed the transient AR reporter gene assay for detecting transcriptional activities via hAR using the CV-1 cell line. The assay displayed appropriate response to the known AR agonist DHT and AR antagonist nilutamide. DHT significantly induce CAT activity at concentration of 10^{-10} M. The maximal induction achieved at 10^{-8} M and leveled off thereafter with EC_{50} value of 3.9×10^{-10} M. DHT induced CAT activity in a concentration-dependent manner. The cell not transfected with AR expression plasmid AR/pcDNA3.1 did not respond to DHT. It was indicated that the activation was through AR. Nilutamide was a potent antiandrogen that exhibited significant antagonistic activity at concentration of 10^{-7} M and greater. The IC_{50} value was 8.90×10^{-7} M. The CAT activity could not be induced by GR agonist dexamethasone, which suggested that the assay system be highly specific to androgenic compounds without cross-talk to GR agonist. The assay showed acceptable repeatability to DHT with intro CV of 9.3% and inter CV of 10.9%. In conclusion, the AR reporter gene assay in the present study provided a promising tool to screen for AR agonists and antagonists.

DDT and its metabolites are still problems of great relevance to public health (Turusov et al., 2002). Studies indicated exposures to DDT affected the reproductive health of male including reduced sperm counts, fertility and increased risk of birth defects (Salazar-Garcia et al., 2004). The reporters from Kelce et al. (1995, 1997) suggested that the effects of DDT on the male reproductive system be mediated by the primary metabolite *p,p′*-DDE though AR. It has been demonstrated that *p,p′*-DDE is potent antiandrogen mediated by AR in vitro and in vivo. Once bound to AR, *p,p′*-DDE is imported into the nucleus and inhibits expression of androgen regulate genes such as TRPM-2 and C3. In the AR reporter gene assay, *p,p′*-DDE was effective inhibitor of androgen induced AR transcriptional activity. In the present study, we demonstrated that *p,p′*-DDE decreased DHT induced CAT activity obviously. The results were in agreement with the previous studies. We failed to find AR agonistic effect of *p,p′*-DDE in this study. Because, little estrogenic activity was detected previously, *p,p′*-DDE acts as ECD predominantly via AR as antagonist.

Fenvalerate, a synthetic pyrethroid insecticide, is widely used in agriculture, particularly in developing countries. Previous study in our laboratory and other studies have shown that several pyrethroid pesticides including fenvalerate exerted estrogenic activities (Chen et al., 2002; Go et al., 1999). The androgenic effects of fenvalerate have received comparatively little attention. Using the AR reporter gene assay, we investigated the androgenic and antandrogenic activities of fenvalerate whose reproductive effects were already demonstrated. In this study, the pesticide did not appear to activate AR-mediated effect. When the AR antagonistic activity was examined in the presence of DHT, fenvalerate appeared to inhibit the activation induced by DHT with IC_{50} of 7.10×10^{-5} M. Fenvalerate was considered to act as weak AR antagonist. The result in the study was confounded by the absent antiandrogenicity of fenvalerate in vivo. The authors were unable to identify fenvalerate to interfere with AR-mediated mechanisms (Kunimatsu et al., 2002). Therefore, the assay in vitro can provide valuable insights into mechanisms of action of test chemicals, but has limited ability to mimic whole animal chemical metabolism and distribution. For better understanding of above consideration, it is necessary to evaluate fenvalerate by in vivo study.

Phoxim is a highly effective organophosphorus pesticide with low toxicity. Phoxim did not show ability to bind to ER and induce estrogen-responsive gene pS2 (Chen et al., 2002). The assay system was used to investigate the AR-mediated effects of phoxim. Neither agonistic nor antagonistic activity was detected, which was different from another organophosphate pesticide fenitrothion. Tamura et al. (2003) reported that fenitrothion was an AR antagonist. The discrimination in chemical structure of the two pesticides may be the primary cause for the discrepancy of antiandrogen activity (Fig. 1). Tamura et al. (2003) suggested an aromatic ring (A-ring) with a strong hydrogen bond (H-bond) acceptor be necessary for AR antagonistic activity. Fenitrothion and other organophosphate pesticides possessing antiandrogenic activities all contain the essential structure. The chemical structure of phoxim containing phenyl ring but without H-bond acceptor might account for the undetectable AR antagonistic activity.

In conclusion, the AR reporter gene assay in the present study is a promising tool to screen for AR

agonists and antagonists. Our data demonstrate that *p,p′*-DDE is potent antiandrogen mediated by AR in vitro. Fenvalerate acts as weak AR antagonist and phoxim dose not show antiandrogenic activity.

Acknowledgements

The authors gratefully acknowledge Dr. M. Takeyoshi for providing the plasmid AR/pcDNA3.1. The project was supported by the Greatest Project in the National Basic Research (No. 2002CB512908) and National Ten-Five Key Technologies R&D Programme (No. 2004BA720A33).

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